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An Evaluation of *Sceptridium dissectum* (Ophioglossaceae) with ISSR Markers: Implications for *Sceptridium* Systematics

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ABSTRACT.—*Sceptridium dissectum*, the most variable North American grapefern species, demonstrates variation in degree of blade dissection, blade color, pinnule shape, and pinnule margins. Historically, various morphologies have been included within *S. dissectum*. For example, Clausen's monograph recognized five infraspecific taxa in *S. dissectum*, of which only the morphologies of variety *dissectum* and *obliquum* are currently retained. However, the taxonomic status of the two varieties has been debated. We used ISSR (Inter-Simple Sequence Repeat) markers to assess the genetic distinctness of *S. dissectum* var. *dissectum* and var. *obliquum* in 17 Ohio populations. Five ISSR primers generated 69 reproducible loci. In UPGMA analyses and AMOVA, *S. dissectum* var. *dissectum* individuals did not cluster separately from var. *obliquum* individuals, nor did individuals from the same population cluster together. ISSR markers revealed levels of population genetic structure in *S. dissectum* similar to levels detected by previous isozyme investigations. Our results concur with recent treatments of *S. dissectum* that do not formally recognize infraspecific taxa, and may bring into question current species circumscriptions in *Sceptridium*. We illustrate the use of ISSR markers for examining taxonomic boundaries in *Sceptridium*.

Species of *Sceptridium* Lyon, the evergreen grapeferns, are common members of temperate and north temperate habitats, though the genus has a worldwide distribution (Wagner and Wagner, 1983). In North America, the center of species diversity lies east of the Mississippi River to the Atlantic Coast, and from the southern Gulf Coast to the northern coasts of the Great Lakes (Wagner and Wagner, 1993). Within this range, Wagner and Wagner (1993) recognized seven species, and it is not uncommon to find more than one species at a single site (Wagner, 1960a). Most *Sceptridium* species inhabit a variety of moderately disturbed habitats such as secondary-growth woods, old fields, and grassy slopes, although some species may occur in more undisturbed habitats (Clausen, 1938).

Species of *Sceptridium*, like other members of Ophioglossaceae, generally produce one epigeal leaf per year, which is divided into a sterile trophophore and a fertile sporophore (Clausen, 1938). Unlike some members of the family (*e.g., Botrychium s.s.*; Wagner, 1990), *Sceptridium* species do not always produce a sporophore, and under stressful conditions may not produce a trophophore (Wagner, 1960b; Montgomery, 1990; Wagner and Wagner, 1993; Kelly, 1994). The leathery, photosynthetic trophophore persists through the winter, hence the moniker "evergreen" grapefern. *Sceptridium* species, as well

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as other members of the family, possess subterranean, non-photosynthetic, mycoparasitic gametophytes (Wagner *et al.*, 1985; Melan and Whittier, 1989). The subterranean nature of the gametophytes may be associated with high self-fertilization rates (Tryon and Tryon, 1982) as has been documented in some *Sceptridium* species (McCauley *et al.*, 1985; Watano and Sahashi, 1992).

TAXONOMIC HISTORY OF *SCEPTRIDIUM* AND *S. DISSECTUM* (SPRENG.) LYON.—*Sceptridium* was first recognized as a genus by Lyon (1905) after observing the embryo morphology of *Botrychium dissectum* var. *obliquum* (Muhl.) Clute. Lyon (1905) found that the embryo of *B. dissectum* var. *obliquum* differed from the embryo of *B. virginianum* (L.) Swartz by possessing a long suspensor that lacked a pronounced lateral cotyledon and a root that emerges from the basal side of the gametophyte. Further, Lyon (1905) noted that most of Underwood's (1898) ternate *Botrychium* species, of which *B. dissectum* var. *obliquum* was included, had a sporophyll that divided into a trophophore and a sporophore near the rhizome. On this basis, Lyon placed most of Underwood's (1898) ternate *Botrychium* species in the genus *Sceptridium*, anticipating each would possess these three characters. Most North American taxonomists have treated *Sceptridium* as a subgenus of *Botrychium* (Clausen, 1938; Lellinger, 1985; Wagner and Wagner, 1993). However, other authors have maintained *Sceptridium* as a separate genus within Ophioglossaceae (Sahashi, 1979; Kato, 1987; Watano and Sahashi, 1992; Hauk, 1996).

Of the seven currently recognized North American *Sceptridium* species, *S. dissectum* is the most variable morphologically (Wagner, 1960b; Wagner and Wagner, 1993). Commenting on *S. dissectum*'s variability, Wagner (1960b) stated that "*Botrychium* [*Sceptridium*] *dissectum* Spreng. is so outlandishly variable that it has apparently misled botanists in delimiting other, closely related, but more uniform, species correctly..." The diversity of blade morphologies encompassed by *S. dissectum* has led to taxonomic disagreement over what range of variation should be included within *S. dissectum*, and what putative segregates deserve recognition as distinct species (Clausen, 1938; Wagner, 1960a; Wagner, 1960b; Wagner, 1961).

Sceptridium dissectum was described by Sprengel in 1804 as *Botrychium dissectum*, and only sporophytes of the more dissected morphology were included in the species by early authors (Sprengel, 1804; Underwood, 1898). Sporophytes possessing relatively less dissected and a more broadly laminated blade morphology were ascribed to *B. obliquum* Muhl. (Underwood, 1898). Variations on these names existed, for example, Prantl (1884) recognized *B. obliquum* and *B. obliquum* var. *dissectum* (Spreng.) Prantl. Until Clausen's monograph (1938), nomenclatural chaos existed concerning the taxonomic limits of *B. dissectum* (for a complete list of synonyms see Clausen, 1938).

In his monograph of the Ophioglossaceae, Clausen (1938) treated *B. dissectum* as four varieties and one subspecies: *B. dissectum* var. *typicum* (*dissectum*), var. *obliquum* (Muhl.) Clute, var. *oneidense* (Gilbert) Farw., var. *tenuifolium* (Underw.) Farw., and subspecies *B. dissectum* ssp. *decompositum* (Mart. & Gal.) Clausen. Of Clausen's five infraspecific taxa, three have been

elevated to species or subsumed into other taxa. Only varieties *dissectum* and *obliquum* (Fig. 1) remain designated as varieties (Clausen, 1938), forms (Wagner, 1960a; McCauley *et al.*, 1985), or not officially recognized but their morphologies mentioned (Lellinger, 1985; Wagner and Wagner, 1993). To have working taxa for analyses and discussion, we followed the nomenclature of Clausen (1938) and considered the two morphologies as varieties.

ISSR PCR.—Multilocus DNA markers have become a useful tool for examining relationships among closely related taxa (Gillies and Abbott, 1998; Kardolus *et al.*, 1998; Parker *et al.*, 1998; Campbell *et al.*, 1999; Nkongolo, 1999; Crawford, 2000; Huang and Sun, 2000; Wolfe and Randle, 2001) because they provide numerous characters derived from multiple sites within the genome (Wolfe and Liston, 1998). ISSR PCR (inter-simple sequence repeat polymerase chain reaction) is a multilocus DNA marker system that has successfully examined relationships among closely related taxa (Wolfe *et al.*, 1998; Huang and Sun, 2000; Culley and Wolfe, 2001; Wolfe and Randle, 2001). Highly variable regions flanking microsatellites are amplified by ISSR PCR primers and minute amounts of genetic variation can be detected (Wolfe and Liston, 1998). When compared to similar techniques such as RAPD (random amplified polymorphic DNA) PCR, ISSR loci are more polymorphic (Kojima *et al.*, 1998; Esselman *et al.*, 1999; McGregor *et al.*, 2000) and reproducible, presumably because of longer primer length and higher annealing temperatures (Nagaoka and Ogihara, 1997; Wolfe and Liston, 1998; Wolfe *et al.*, 1998).

In the present study, we present an investigation of taxonomic boundaries within *Sceptridium dissectum* by comparing inter-simple sequence repeat (ISSR) marker patterns of *S. dissectum* var. *dissectum* and var. *obliquum*. We chose ISSR PCR to 1) assess the genetic distinctness of *S. dissectum* var. *dissectum* and var. *obliquum*, 2) examine *S. dissectum* population genetic structure, and 3) evaluate the utility of ISSR PCR for studying *Sceptridium* taxa.

Materials and Methods

Individual sporophytes were sampled from 17 *Sceptridium dissectum* populations in Ohio (Fig. 2, Table 1). Ten *S. dissectum* var. *dissectum* and 52 *S. dissectum* var. *obliquum* sporophytes were collected. Individuals were selected to represent the range of morphological variation present at each site. Five sporophytes were collected for nine populations, whereas all sporophytes (<5) were collected from eight smaller populations (Table 1). Leaf material from each individual was dried in silica gel for DNA extraction, and the remaining laminar material was pressed. Vouchers were deposited at the Willard Sherman Turrell Herbarium at Miami University (MU).

Total genomic DNA was extracted from approximately 100 mg of silica gel dried leaf material using Qiagen's DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA). Genomic DNA from each sporophyte was quantitated fluorometrically using the PicoGreen dsDNA quantitation reagent (Molecular Probes, Inc., Eugene, OR) and a TD-360 mini-fluorometer (Turner Designs, Sunnyvale,

FIG. 1. Pinnae of *S. dissectum* var. *obliquum* (top) and *S. dissectum* var. *dissectum* (bottom).

CA). Quantitations were performed according to the manufacturer's protocol (Molecular Probes, Inc., Eugene, OR). Each sporophyte's DNA was quantitated twice, and the mean concentration was calculated.

ISSR PCR primers were selected from the University of British Columbia Biotechnology Laboratory (UBC) primer set #9 (Vancouver, BC, Canada: http://www.biotech.ubc.ca). Ninety ISSR primers in the UBC set were screened using DNA from two *Sceptridium dissectum* sporophytes (O-1c & O-1d). We selected the five primers that produced the most robust and clear amplification profiles during primer screening (Table 2).

The ISSR PCR reaction mixture included one unit of *Taq* DNA polymerase, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (all PCR reagents from TaKaRa Shuzo, Co., Ltd, Shiga, Japan) and 0.3 μM of a single ISSR primer with

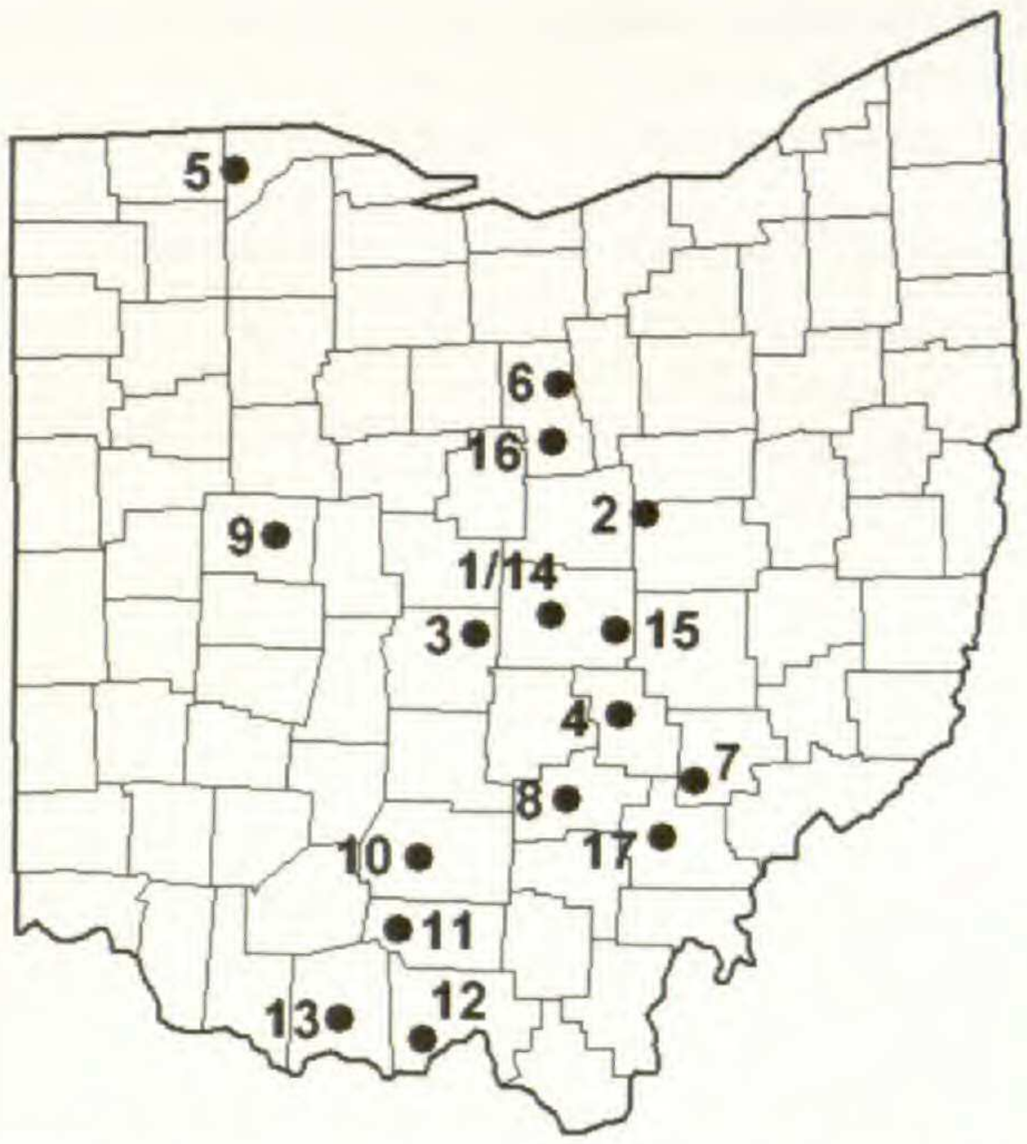

FIG. 2. Distribution of 17 Ohio *Sceptridium dissectum* populations. Numbers correspond to population codes in Table 1.

10 ng of DNA template in a total volume of 25 μL. Reactions were performed in Eppendorf Mastercycler Personal thermalcyclers (Eppendorf AG, Hamburg, Germany) using the following temperature regime: 94°C for 60 seconds, then 35 cycles consisting of 45 seconds at 94°C, 45 seconds at 55°C, and 90 seconds at 72°C followed by a final 5 minute, 72°C extension.

Each ISSR PCR reaction was repeated twice, with appropriate controls, to ensure consistent ISSR profiles. Using a 1 kb Plus DNA ladder size standard (Gibco-BRL, Life Technologies, Inc., Rockville, MD), PCR products were separated electrophoretically at 80 volts for seven hours on 2% agarose gels in TBE buffer with 0.2 ng/mL EtBr. Bands were visualized on a UV trans-illuminator and photographed using a Polaroid MP-4 Land camera (Polaroid Corporation, Cambridge, MA).

ISSR bands were scored from gel photographs. The relatively high annealing temperature (55°C) helped ensure that ISSR bands were reproducible among reactions. Only clear and consistently reproducible bands were scored. Bands of indistinguishable mobility between lanes were assumed to be homologous, and to represent a single ISSR locus. For each sporophyte, each locus was scored as present or absent ("1" = locus present, "0" = locus absent). Data were compiled into a Nexus data matrix using MacClade 4.0 (Maddison and Maddison, 2000).

ISSR loci data were examined with three types of analyses: 1) primer banding profiles, 2) UPGMA (Unweighted Pair Group Method using Arithmetic averages) cluster analyses, and 3) AMOVA (Analysis of MOlecular VAriance; Excoffier *et al.*, 1992). For all analyses we assumed ISSR locus variation was representative of overall genetic variation.

TABLE 1. Population codes, locations, sample sizes, and voucher numbers for Ohio *Sceptridium dissectum* collections. Note that four populations contained both varieties. Vouchers deposited at MU. D = *S. dissectum* var. *dissectum* and O = *S. dissectum* var. *obliquum*.

Population code	Location (County)	Sample size	Voucher
O-1	Licking	5	Barker #70
O-2	Coshocton	5	Barker #85
D-3	Franklin	1	Barker #86
O-3	Franklin	4	Barker #129
O-4	Perry	5	Barker #103
D-5	Lucas	2	Barker #107
O-5	Lucas	3	Barker #130
D-6	Richland	2	Barker #109
O-6	Richland	2	Barker #131
O-7	Morgan	4	Barker #112
O-8	Hocking	2	Barker #113
O-9	Logan	3	Barker #115
O-10	Ross	5	Barker #121
O-11	Pike	5	Barker #123
O-12	Scioto	5	Barker #127
D-13	Adams	1	Barker #128
O-13	Adams	4	Barker #136
D-14	Licking	1	Barker #84
D-15	Licking	1	Barker #95
D-16	Richland	1	Barker #108
D-17	Athens	1	Barker #110

Primer banding profiles were analyzed to assess the utility of ISSR PCR in *Sceptridium*, and to examine the relationship between the two varieties. Banding profiles generated by each primer were examined for the following parameters: 1) variety-specific markers (loci present in >25% of one variety, but in only a few individuals of the other variety; Wolfe *et al.*, 1998), 2) percent of polymorphic loci, 3) number of loci per primer, and 4) number of unique multilocus genotypes per primer. Mean loci and mean multilocus genotypes were also calculated.

UPGMA cluster analysis was used to investigate the distinctness of *S. dissectum* var. *dissectum* and var. *obliquum*, and to examine *S. dissectum* population genetic structure. A phenetic rather than parsimony–based method was used for cluster analyses because we did not verify that all co-migrating loci were homologous or that they sorted independently. Distance matrices were constructed from Dice (1945) and Jaccard (1908) similarity coefficients for UPGMA cluster analysis (Numerical Taxonomy System (NTSYSpc) ver. 2.1t; Rohlf, 2000), and were based only on the shared presence of loci. The absence of an ISSR locus is not informative because any number of non-homologous mutations may result in the loss of a band. Coefficients that calculate distance from both presence and absence of loci are, generally, not appropriate for ISSR data analyses (Wolfe and Liston, 1998). Support for UPGMA clusters was calculated (WinBoot; Yap and Nelson, 1996) with 1000 bootstrap iterations of the data (Felsenstein, 1985).

TABLE 2. Sequences of the ISSR primers used in this study.

Primer	Sequence (5′–3′)	Length (bp)
UBC-818	CAC ACA CAC ACA CAC AG	17
UBC-824	TCT CTC TCT CTC TCT CG	17
UBC-835	AGA GAG AGA GAG AGA GYC	18
UBC-846	CAC ACA CAC ACA CAC ART	18
UBC-880	GGA GAG GAG AGG AGA	15

AMOVAs were conducted as an alternative assessment of the relationship between the *S. dissectum* varieties, and of *S. dissectum* population genetic structure. Distance matrices for AMOVA were generated (Arlequin 2.001, Schneider *et al.*, 2001) as described by Huff *et al.* (1993). The statistical significance of AMOVA results were calculated by a non-parametric permutational analysis of a null distribution for the variance component. To assemble the null distribution of a variance component, individuals are randomly assigned to populations while the number of populations and population sizes are retained from the main analysis (Excoffier *et al.*, 1992). The *P*-value calculated from the null distribution represents the probability of obtaining a larger variance component than the observed values by chance alone. In biological terms, a small *P*-value indicates a low probability of identifying more genetic structure than measured in the observed distribution of individuals, and a high probability of recording less genetic structure. Thus, AMOVA *P*-values only reflect the probability of finding more genetic structure, and do not indicate the biological significance of the observed quantities of genetic structure. In our AMOVAs, null distributions were generated with 1023 permutations of the data (Arlequin 2.001, Schneider *et al.*, 2001).

AMOVA was also used to calculate an F_{ST} value for the distribution of *S. dissectum* population genetic variation. For dominant marker data (e.g., ISSR or RAPD), the F_{ST} value calculated by AMOVA is a correlation of genotypes rather than individual co-dominant sites, as in isozymes. Further, identical breeding mechanisms were assumed for all *S. dissectum* populations. Thus, an F_{ST} value calculated from dominant marker data may not be directly comparable to F_{ST} values generated from co-dominant marker data.

RESULTS

Five ISSR primers produced 69 loci (mean = 13.8/primer) with 94% of the loci polymorphic (Table 3). Primer UBC-818 produced the most loci (16), whereas primer UBC-846 produced the fewest (12). The mean number of unique multilocus genotypes distinguished per primer was 38.2 (Table 4). No variety-specific markers were identified. For each primer surveyed, some individuals of *Sceptridium dissectum* var. *dissectum* possessed banding profiles identical to those of some var. *obliquum* individuals. All individuals were distinguished as unique multilocus genotypes using a combination of any

TABLE 3. Sample size, total number of loci, and percent polymorphic loci generated by five primers from the ISSR survey of *S. dissectum* var. *dissectum* and var. *obliquum*.

Taxon	Sample size	Total # loci	% Polymorphic loci
S. dissectum var *dissectum*	10	50	82%
S. dissectum var. *obliquum*	52	62	92%
Total	62	69	94%

three primers, and using all five primers, the genotypic diversity (# genotypes/ # individuals) for each taxon was 1.0.

Our investigation of *S. dissectum* population genetic structure revealed that individuals from the same *S. dissectum* population did not cluster closely in UPGMA analyses (Figs. 3 & 4). Most clusters consisted of individuals from different, and sometimes, distant populations. For example, O-9a, collected in Logan County in west central Ohio, clustered with O-6d, a specimen from Richland County in north central Ohio, approximately 120 miles away. This pattern was repeated for other individuals from geographically distant sites, but for the clusters supported by bootstrap values >50%, all individuals were from the same population (Figs. 3 & 4). Seven clusters consisted of two individuals from the same population, and, of these, only O-1c + O-1d + O-1e and O-12a + O-12b were supported by bootstrap values >50% (Figs. 3 & 4). In neighbor joining (NJ) or maximum parsimony (MP) analyses (not presented), populations did not form discrete clusters. The NJ and MP tree topologies were essentially identical to the UPGMA topologies (Figs. 3 & 4), and had equivalent levels of bootstrap support.

AMOVA also revealed little genetic structure among *S. dissectum* populations. Of the total genetic variation detected, among-population genetic variation was 8.49%, whereas within-population was 91.51%. A low level of genetic structure for the *S. dissectum* populations was also indicated by the F_{ST} value (0.085, $P < 0.0001$, Table 5). The highly significant *P*-value suggests that our observed distribution of individuals in populations produces nearly the largest amount of genetic structure possible in our data set.

In a comparison of the two *S. dissectum* varieties, neither var. *dissectum* nor var. *obliquum* formed discrete clusters in UPGMA analyses (Figs. 3 & 4). Individuals of var. *dissectum* frequently clustered more closely with members of var. *obliquum* than with their own taxonomic group. Bootstrap support for all but three clusters in the UPGMA trees (Figs. 3 & 4) was poor. The two varieties failed to form discrete clusters in NJ and MP analyses (not presented) which had almost identical tree topologies and similar levels of bootstrap support.

AMOVA revealed little genetic difference between *S. dissectum* var. *dissectum* and var. *obliquum*. The two varieties were only 3.38% genetically different, while they were 96.62% genetically similar (Table 6). The amount of genetic difference identified between the two varieties was close to the largest amount possible in our data set ($P = 0.0140$, Table 6).

TABLE 4. Number of loci and genotypes distinguished for each primer. Because some sporophytes of each variety were indistinguishable when examined with a single primer, # loci and genotype values are the combined result for both *S. dissectum* varieties.

Primer	# Loci	# Genotypes
UBC-818	16	55
UBC-824	14	51
UBC-835	13	36
UBC-846	12	27
UBC-880	14	22
Mean	13.8	38.2
Total	69	62

DISCUSSION

Marker systems used to examine relationships among species or subspecific taxa should provide highly variable loci, and the system should be able to distinguish as many individuals of a single species as possible, in concordance with the organism's breeding system (Avise, 1994). In the present study, ISSR markers distinguished all *S. dissectum* individuals by any combination of three primers, a result similar to that observed in other studies (Wolfe *et al.*, 1998; Esselman *et al.*, 1999). Of the ISSR loci distinguished in our *S. dissectum* taxa, 82% (var. *dissectum*), 92% (var. *obliquum*) and 94% (species total) were polymorphic (Table 3), values well within the range of ISSR variability when ISSR markers have successfully discriminated taxa at the species level and lower (Wolfe *et al.*, 1998; Culley and Wolfe, 2001; Wolfe and Randle, 2001). For example, Wolfe *et al.* (1998) demonstrated patterns of diploid hybrid speciation in *Penstemon* using ISSR markers, and reported percent polymorphic loci values of 72–95% for the seven taxa sampled. Wolfe and Randle (2001) used ISSR markers to examine taxonomic boundaries and relationships in *Hyobanche* and found that 64–96% of their ISSR loci were polymorphic in four taxa. ISSR markers discriminated between two varieties of *Viola pubescens* with 100% of ISSR loci polymorphic for the species (Culley and Wolfe, 2001). As values for ISSR variability in *S. dissectum* were within the range reported from studies that have successfully used ISSR markers to examine taxonomic boundaries and relationships, ISSR markers appear to be an appropriate tool for examining taxonomic boundaries among *Sceptridium* subspecific taxa and possibly species.

DISTRIBUTION OF GENETIC VARIATION.—As assessed by ISSR genotypes, the distribution of genetic variation in *S. dissectum* was consistent with results from studies of other pteridophyte species, where most genetic variation was distributed within populations (Haufler and Soltis, 1984; Holsinger, 1987; Kirkpatrick *et al.*, 1990; Soltis and Soltis, 1987; Soltis *et al.*, 1988; Soltis and Soltis, 1988; Watano and Sahashi, 1992). Using ISSR PCR, Camacho and Liston (2001) found most genetic diversity partitioned within populations of *Botrychium pumicola*, and little among population genetic differentiation. Within *Sceptridium*, Watano and Sahashi (1992) reported that 81% of isozyme

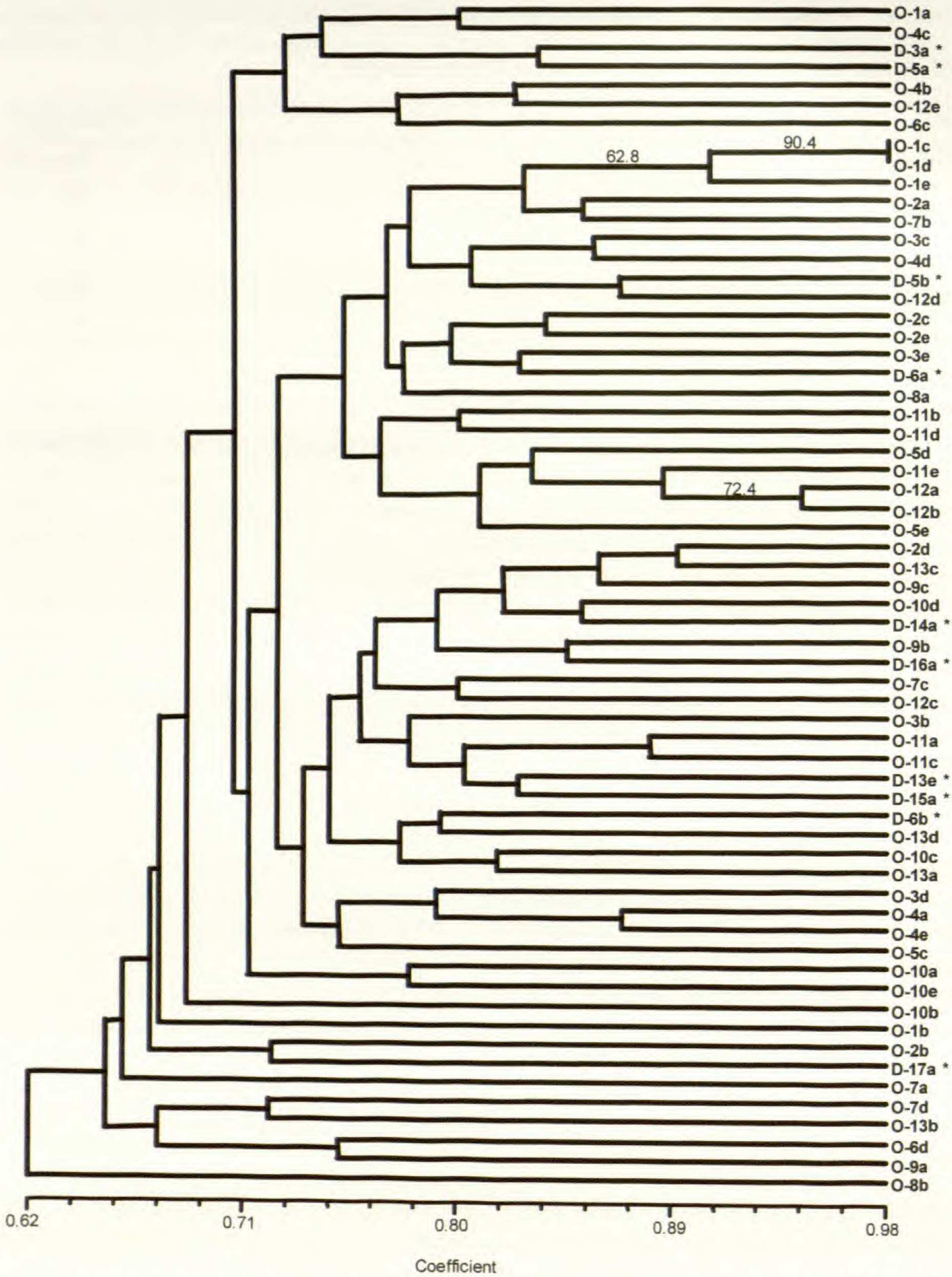

FIG. 3. UPGMA cluster analysis of 62 *Sceptridium dissectum* sporophytes based on 69 ISSR loci generated from five ISSR primers using a distance matrix generated with the Dice (1945) algorithm. Bootstrap values >50% are reported above branches. The scale below the dendogram refers to the coefficient of similarity represented by corresponding branch lengths. Labels correspond to population codes in Table 1, asterisks indicate var. *dissectum* individuals, and lowercase letters (*i.e.*, a, b, c, d, e) distinguish individuals of the same population.

FIG. 4. UPGMA cluster analysis of 62 *Sceptridium dissectum* sporophytes based on 69 ISSR loci generated from five ISSR primers using a distant matrix generated with the Jaccard (1908) algorithm. Bootstrap values >50% are reported above branches. The scale below the dendogram refers to the coefficient of similarity represented by corresponding branch lengths. Labels correspond to taxon–population codes in Table 1, asterisks indicate var. *dissectum* individuals, and lowercase letters (*i.e.*, a, b, c, d, e) distinguish individuals of the same population.

TABLE 5. AMOVA statistics for *S. dissectum* population genetic structure based upon ISSR marker profiles. *P* is the probability of obtaining a larger variance value.

Variance component	Variance	% Total variation	F_{st}	*P*
Among-populations	1.240	8.49	0.085	<0.0001
Within-populations	13.36	91.51	—	—

allelic genetic diversity was distributed within and 19% was distributed among *S. ternatum* populations. Based upon similar isozyme data, McCauley *et al.* (1985) estimated an F_{ST} value of 0.090 for *S. dissectum* var. *obliquum* populations, similar to our ISSR-based value of 0.085. *Sceptridium dissectum* genetic variation, as measured by ISSR genotype distribution, is within the range for isozyme allelic distribution reported by Soltis and Soltis (1990) for both outcrossing and inbreeding fern species. Thus, our ISSR data are consistent with previous evidence from ISSR and isozyme studies concerning the distribution of genetic variation.

UPGMA cluster analyses revealed most *S. dissectum* individuals (*i.e.*, genotypes) did not group by population, but, rather, individuals from disparate populations often grouped together. Camacho and Liston (2001) found that cluster analysis of ISSR data did not segregate by population individuals of the related and presumably inbreeding *Botrychium pumicola*. Other, similar ISSR studies have detected population genetic structure that was evident in cluster analyses (Wolfe *et al.*, 1998; Culley and Wolfe, 2001). Among five populations of *Viola pubescens* var. *scabriuscula* Schwein., populations were clearly defined by cluster analysis (Culley and Wolfe, 2001). We generated a similar number of scorable loci as reported by Culley and Wolfe (2001). Thus, if *S. dissectum* populations are truly differentiated genetically, the amount of ISSR data generated in our study should have revealed it, especially because a high proportion of individuals at each site was collected.

Initially, the inability of UPGMA to cluster *S. dissectum* ISSR genotypes by population may appear contrary to published isozyme studies. Watano and Sahashi (1992) reported only two isozyme genotypes were shared among three populations of *S. ternatum*, indicating the three populations were markedly dissimilar in genotypes and suggesting individuals within populations should be of similar genotype. However, when Watano and Sahashi (1992) measured allelic diversity, 81% of the genetic variation was distributed within populations. Thus, allelic diversity and genotype distribution in *S. ternatum* did not produce similar estimates of the distribution of genetic variation. The apparent discrepancy between allelic diversity and genotype distribution in *S. ternatum* may be a consequence of founder effects, selection, genetic drift (Watano and Sahashi, 1992), or the total amount of variation detected. If more isozyme genotypes had been detected, then estimates of allelic diversity and genotype distribution may have been more similar. Watano and Sahashi (1992) identified only 30 genotypes from 138 *S. ternatum* individuals, whereas ISSR markers identified 62 genotypes from 62 *S. dissectum* individuals. Based on ISSR data, populations of congener *S. dissectum* do not frequently consist of

TABLE 6. AMOVA statistics from a comparison of the ISSR profiles of *S. dissectum* var. *dissectum* and var. *obliquum*. *P* is the probability of obtaining a larger variance value.

Variance component	Variance	% Total variation	*P*
Between-taxa	0.5013	3.38	0.0140
Within-taxa	14.34	96.62	—

individuals of identical or similar genotypes, although this does not exclude the possibility that the populations may contain similar isozyme genotypes, as observed by Watano and Sahashi (1992) in *S. ternatum*. Further, ISSR measures of genotype distribution in *S. dissectum* are more similar to overall estimates of fern isozyme allelic diversity (Soltis and Soltis, 1990) than to the distribution of isozyme genotypes as reported by Watano and Sahashi (1992). Other populations of inbreeding pteridophytes studied previously with isozymes should be surveyed with ISSR PCR to determine if genotype distributions similar to ours can be documented.

The rather low partitioning of genetic variation within and among fern populations has been explained by high rates of spore dispersal, rapid colonization of a region with little subsequent genetic differentiation, or both (Soltis and Soltis, 1988; Soltis and Soltis, 1990). In *S. dissectum* either scenario is possible, and probably a combination of both has contributed to the current distribution of genetic variation. During the latest glaciation event (Wisconsian), ending approximately 15,000 y.a. (Smith and Smith, 2001), *S. dissectum* may have been restricted to the Southern Appalachians and the Gulf Coast. Recolonization of deglaciated areas with insufficient time for subsequent genetic differentiation of populations may have contributed to the observed distribution of genetic variation. Alternatively, spore dispersal in *S. dissectum* may be high enough to effectively link the sampled populations to form a large metapopulation, which may account for the present distribution of genotypes. Based on ISSR data, it is impossible to exclude either rapid colonization or spore dispersal as the primary cause of the observed distribution of genetic variation in *S. dissectum*.

Dominant marker systems, such as ISSR PCR, are inappropriate for estimating self-fertilization rates, and inferring an organism's breeding system. Although ISSR markers may provide enough resolution to distinguish many or all individuals in a population, the technique does not provide a measure of true heterozygosity, a requirement for estimating self-fertilization rates (Wolfe and Liston, 1998). As such, we were unable to determine the breeding system of the *S. dissectum* populations sampled. However, the isozyme studies of McCauley *et al.* (1985) and Watano and Sahashi (1992) demonstrated that high rates of self-fertilization characterize the breeding system of the *Sceptridium* species studied. Our results cannot support or refute the results of these isozyme studies.

TAXONOMIC IMPLICATIONS OF ISSR DATA.—Analyses of ISSR marker data demonstrated no ISSR loci specific to either *Sceptridium dissectum* var.

dissectum or *S. dissectum* var. *obliquum*. If the two taxa were genetically distinct, each taxon should have unique ISSR loci. Moreover, individuals from each variety should form discrete clusters in UPGMA, and this did not occur (Figs. 3 & 4). The lack of genetic distinction between the two varieties is illustrated by relationships between individuals D-6a and O-3e. Both individuals clustered more closely to each other than to any other individual in the data set, but, morphologically, D-6a represents var. *dissectum* and O-3e represents var. *obliquum*. The AMOVA comparison of genetic variation between the *S. dissectum* varieties also demonstrated that the taxa were genetically indistinguishable, sharing 96.62% of their ISSR genetic variation. These ISSR results support Tryon's (1936) observations of a few var. *dissectum* individuals producing var. *obliquum* fronds (and vice versa) in subsequent seasons. If *S. dissectum* trophophore morphology truly exhibits such seasonal plasticity, then the clustering of var. *dissectum* individuals with var. *obliquum* individuals would be expected, and both ISSR data and Tryon's (1936) observations indicate no genetic distinctness between the two varieties.

Although the UPGMA analyses did not separate the two *S. dissectum* varieties, most groups were poorly supported by bootstrap analysis. The lack of bootstrap support for most groups generated in the UPGMA cluster analyses may be a result of primer to primer variation, *e.g.*, O-8b clustered with D-13e using primer 835, but with O-5e using primer 824. The lack of consistent relationships among ISSR primers in *S. dissectum* may be due to the scoring of non-homologous ISSR loci. Available evidence from other studies (S. Datwyler, Ohio State Univ., pers. comm.) suggests that this is an unlikely source of the inconsistencies observed. Shannon Datwyler (pers. comm.) examined ISSR loci from 30 different Scrophulariaceae species and established estimates of ISSR locus homology. For high frequency bands (present in >6 individuals) in *Penstemon*, she reported 83% of the bands scored as homologous were homologous as determined by Southern hybridization. In *Scrophularia* and *Hyobanche*, 93% and 100% of co-migrating ISSR bands, respectively, were homologous (Datwyler, pers. comm.). In our data set, 97% of scored loci were considered high frequency by Datwyler's criterion, but we have not verified the homology of scored bands. Additionally, in *Helianthus* and *Brassica*, Adams and Rieseberg (1998) found that even when 20% of the bands in a RAPD PCR data set were non-homologous, there was negligible effect on species relationships as generated by principal-coordinate-analysis ordination. If these findings in *Brassica* and *Helianthus* can be extrapolated to ISSR cluster analyses in *Sceptridium*, then even a substantial number of non-homologous bands may have no significant impact on relationships among individuals.

Another possible cause for the low UPGMA bootstrap support and primer to primer variation was the nature of ISSR loci variation. In the data matrix containing the calculated genetic similarity values for the *S. dissectum* ISSR results (not presented), some individuals were equally similar to other individuals, although their banding patterns were all unique. Because UPGMA clusters individuals by seeking combinations of the least different similarity

values (Avise, 1994), the few sets of identical genetic similarity values may have caused the UPGMA algorithm to make arbitrary decisions between individuals when clustering (Takezaki, 1998), resulting in the production of tie trees during bootstrap analysis. The production of tie trees can lower bootstrap support for UPGMA clusters (Takezaki, 1998), and this may explain the low bootstrap support in the ISSR UPGMA analyses. After close examination, the low bootstrap values for the UPGMA analyses do not discredit the interpretation that the two varieties are not different, but further support this conclusion as genetic similarity values between individuals of the two varieties were frequently equivocal with similarity values between individuals of the same variety.

Wagner (1960a) argued that if two putative taxa co-exist over large areas with intergradation in morphological characters between the taxa, then the two entities should not be recognized. Our ISSR data provide evidence that no underlying genetic differentiation correlates with the morphologies of *S. dissectum* var. *dissectum* and var. *obliquum*, and recognition of varieties with formal taxonomic status in *S. dissectum* is not supported. Because taxonomic designations based upon morphology often imply genetic distinctness (Paris *et al.*, 1989), formal recognition of var. *dissectum* and var. *obliquum* may perpetuate this assumption. Based on the available ISSR evidence and Wagner's (1960a) criteria for varieties, var. *dissectum* and var. *obliquum* should not be recognized as formal taxonomic units. More recent classifications that do not formally recognize infraspecific variation in *S. dissectum* (*e.g.*, Lellinger, 1985; Wagner and Wagner, 1993) reflect more clearly the genetic evidence at hand than do earlier classification systems (*e.g.*, Clausen, 1938).

Morphology alone apparently does not accurately depict genetic relatedness among individuals of the highly variable *S. dissectum*. A logical extension of these data calls into question species level taxonomy in *Sceptridium* that is based solely on variable morphological characters. ISSR markers have proven useful for examining species level distinctions in angiosperms (Wolfe *et al.*, 1998; Wolfe and Randle, 2001) and may be useful for examining relationships among *Sceptridium* species. For example, preliminary ISSR data suggest that *S. oneidense* (Gilb.) Lyon, a taxon previously included as a variety of *S. dissectum*, is not genetically distinct from *S. dissectum*, whereas *Botrypus* (=*Botrychium*) *virginianus* (L.) Michx. is distinct at many loci (Barker and Hauk, unpubl. data). If ISSR markers reveal other *Sceptridium* species closely related to *S. dissectum* as genetically indistinguishable, then a critical reexamination of species concepts in *Sceptridium* is warranted.

The large range of morphological variation in *Sceptridium* species may be the consequence of two different phenomena. First, *Sceptridium* species possess some of the highest reported self-fertilization rates among vascular plants (McCauley *et al.*, 1985; Watano and Sahashi, 1992). The high self-fertilization rate may be a source of morphological variation among self-fertilizing lineages through within-lineage fixation of genes controlling laminar characters. For example, Schneller and Holderegger (1997) reported

inbred progeny of *Athyrium filix-femina* (L.) Roth demonstrated "considerable morphological variation" over outcrossed progeny. Another possible explanation for morphological variation in *Sceptridium* may be phenotypic plasticity affected by various environmental conditions. This may explain the differences observed by Tryon (1936) between *S. dissectum* var. *dissectum* and var. *obliquum* (*i.e.*, individuals producing blades with either morphology in different years), and ISSR markers did not reveal any genetic differences between the two varieties. Combined with other data sources (isozymes, RFLPs, DNA sequences, etc.) ISSR markers should be useful for examining critically species delimitations in *Sceptridium*, and may contribute to a better understanding of morphological variation in the genus.

Conclusions

ISSR markers proved useful for examining infraspecific genetic variation in *S. dissectum* by distinguishing all individuals and producing levels of polymorphic loci within the range reported by similar ISSR studies. The low level of population genetic structure detected by ISSR markers in *S. dissectum* populations was consistent with previous isozyme studies of *S. dissectum* and other fern species. Morphologies traditionally identified as var. *dissectum* and var. *obliquum* did not correlate with ISSR marker variation, and our data do not support the recognition of these as infraspecific taxa. Species boundaries in *Sceptridium* should be critically examined because morphological distinctions among the species are not always clear.

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